*INDEPENDENT VARIATION IN NATURE OF HEMAGGLUTININ AND NEURAMINIDASE ANTIGENS OF INFLUENZA VIRUS: DISTINCTIVENESS OF HEMAGGLUTININ ANTIGEN OF HONG KONG/68 VIRUS**

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Abstract.—Antigenic variations of the two virus-coded surface proteins of influenza virus—hemagglutinin and neuraminidase—were examined in seven strains of influenza A_2 virus (including the Hong Kong/68 strain) isolated from 1957 to 1968. Changes in the two antigens were found to occur independently in nature, resulting in new viruses which differ from older strains more with respect to one antigen than the other. The Hong Kong/68 strain is markedly different from previous A_2 strains in its hemagglutinin antigen but possesses neuraminidase indistinguishable antigenically from that of recent strains. Immunization experiments in mice provided evidence that only the neuraminidase component of an earlier A_2 strain provided protection against Hong Kong virus challenge.

Segregation of hemagglutinin and neuraminidase antigens through recombination of each of the seven strains of influenza A_2 virus with A_0/NWS virus made it possible to investigate antigenic variation of the two dissociated surface proteins independently in a common plaque assay system. Comparison of these hybrid viruses with the parent A_2 strains provided evidence that all the cross-reactivity of the Hong Kong strain with previous A_2 viruses is explicable on the basis of its similar neuraminidase component. It is proposed that the taxonomy of influenza A viruses must take into account differences in neuraminidase as well as hemagglutinin antigens.

Assessment of changes in antigenic structure and the classification of strains of influenza A viruses into subtypes have been based principally on similarities and differences among hemagglutinin proteins and measurements reflected by neutralization and hemagglutination-inhibition tests.

However, influenza virus possesses in its envelope a second virus-coded protein antigen, neuraminidase, which is chemically and immunologically distinct from hemagglutinin.[1–3] Antibody specific for viral neuraminidase is produced after infection or artificial immunization of man[4] or laboratory animals,[5] and recent experiments in this laboratory have provided evidence that anti-neuraminidase antibody may have an important function in immunity to influenza.[5]

The antigenic dissimilarity of A_0 and A_2 enzymes[2] provided valuable markers in recombination studies and led to the recognition of antigenic hybrids of A_0/NWS and A_2/RI/5, and of A_0/NWS and A_1/CAM viruses.[6–8]

More recent experiments have suggested antigenic similarities in the enzyme antigens of strains of influenza A_0 and influenza A_1 viruses as well as

differences in the neuraminidase antigens among strains of influenza A_2 virus.[9, 10] However, these studies did not attempt to assess quantitatively the antigenic changes of neuraminidase and hemagglutinin in a common assay system in which specific inhibition of the biologic activities of these proteins could be measured. In the present report, antigenic analysis of test viruses by plaque assay in clone 1-5C-4 cells permitted separate comparisons of antigenic similarities of neuraminidase and hemagglutinin proteins in a single system. In this system, antibody is incorporated into the agar overlay after infection of the monolayer with influenza virus; antibody directed against hemagglutinin inhibits completely the formation of plaques, and antineuraminidase antibody reduces the size but not the number of plaques.[11]

We conducted a series of experiments in which antigenic variations of neuraminidase and hemagglutinin proteins were compared in seven strains of influenza A_2 virus isolated in the 1957–1968 period, including the new Hong Kong variant. (Throughout this report the Hong Kong/68 virus will be referred to as an "A_2" strain in keeping with common practice. However, the conclusions drawn from the present study raise questions as to the validity of this classification.) The purpose of our investigations was to determine whether antigenic changes in the two surface antigens evolved simultaneously or independently in nature, and whether the epidemiology of influenza might be influenced by antigenic changes in neuraminidase. In particular, we were interested in comparing neuraminidase and hemagglutinin antigens of the so-called Hong Kong (1968 variant) virus to those of earlier strains. The independent genetic control of the neuraminidase and hemagglutinin proteins had been clearly established by genetic recombination of A_0 and A_2 viruses in this laboratory and isolation and separation of the virus proteins by disruption of the virus and electrophoresis.[3]

The present antigenic analysis was facilitated by segregation of hemagglutinin and enzyme antigens through recombination of each of the seven strains with A_0/NWS virus by methods reported earlier.[8] This report will be primarily concerned with studies of hybrid viruses containing the neuraminidases of the parental A_2 strains, and the hemagglutinin of A_0/NWS virus. Experiments in progress utilize reciprocal hybrid viruses that possess the hemagglutinin antigens of the A_2 viruses and the neuraminidase of A_0/NWS virus.

Materials and Methods.—Viruses: Chick embryo allantoic fluid seeds of the following viruses were employed: A_2/Jap.305/57; A_2/Taiwan/1/64; A_2/Itsukaichi/1/65; A_2/Montevideo/2208/67; A_2/England/10/67; A_2/Texas/2/68 and A_2/Hong Kong/16/68. (We are grateful to the Communicable Disease Center, Atlanta, for supplying these strains to us.) Each strain was hybridized with A_0/NWS virus by methods described in detail elsewhere,[8] producing viruses that contained the hemagglutinin antigen of A_0/NWS and the neuraminidase of the test strain. In addition, the following recombinant viruses which have been described previously[8] also were employed: X-7 (hemagglutinin of A_0/NWS, neuraminidase (E), derived from A_2/RI/5$^+$/1957); X-9 (hemagglutinin from A_2/RI/5$^-$, neuraminidase (e) from A_0/NWS). These recombinant viruses have been given the antigenic designations indicated in Table 1. One additional recombinant employed was X-15 virus (A/Equi$_1$ hemagglutinin, neuraminidase of A_2/RI/5$^+$). This virus has the unusual property of reacting with antibody specific for A_2 virus neuraminidase in hemagglutination-inhibition tests, and antibody to X-15 virus inhibits hemagglutination by viruses possessing the A_2 enzyme.[12]

TABLE 1. *Antigenic designations of recombinant viruses prepared from influenza A_0/NWS and influenza A_2 viruses isolated from 1957 to 1968.*

Recombinant	Hemagglutinin antigen	Enzyme antigen
A_0E (J.305)	A_0/NWS/33	A_2/Jap.305/57
A_0E (Taiwan)	"	A_2/Taiwan/64
A_0E (Itsuk.)	"	A_2/Itsukaichi/65
A_0E (Mont.)	"	A_2/Montevideo/67
A_0E (Eng.)	"	A_2/England/67
A_0E (Texas)	"	A_2/Texas/68
A_0E (H.K.)	"	A_2/Hong Kong/68
A_0E (RI/5)*	"	A_2/RI/5^+/57
A_2e (NWS)†	A_2/RI/5^-/57	A_0/NWS/33

* X-7 in earlier reports.[8]
† X-9 in earlier reports.[8]

Antisera: Test viruses were partially purified by elution from red cells, adjusted to similar hemagglutination titers, mixed in an equal volume of Freund's adjuvant, and injected into the footpads of rabbits. A second injection was given 40 days later and serum specimens were obtained 7 days after the second injection.

Hemagglutination-inhibition: Tests were performed in tubes with serial twofold dilutions of sera inactivated by heat and KIO_4 according to standard methods.[13]

Enzyme-inhibition: Enzyme assays and enzyme-inhibition tests were performed by a modification of the Warren method with a fetuin substrate. Allantoic fluid virus suspensions employed as the enzyme source were adjusted to liberate sufficient *N*-acetyl neuraminic acid to give optical density readings of 400–600 at 549 mμ in a Beckman DU-2 spectrophotometer. Titers of antisera are expressed as that dilution which inhibited 50% of the neuraminidase activity of the test virus.

Mouse challenge: Male MF-1 mice (12 to 16 weeks old) previously immunized by injection of ultraviolet-inactivated virus mixed in complete Freund's adjuvant were challenged with approximately 100 ID_{50} of virus by aerosol. Pulmonary virus titers were measured 2 days after challenge, and lung lesions were scored 7 days after challenge, according to methods described previously.[14]

Cells and plaque assay: Clone I-5C-4 derived from the Wong-Kilbourne variant of the human aneuploid Chang conjunctional cell line was used for tests for plaque inhibition and for plaque-size reduction. The methods for assay of plaques and for inhibition of plaques with antisera have been described in detail.[7, 11]

Results.—(a) *Antigenic changes in neuraminidase:* Rabbit antiserum to purified neuraminidase derived from a 1957 strain of A_2 virus[15] was titrated in enzyme-inhibition (EI) tests against homologous virus and against each of the other strains isolated from 1957 to 1968. The results shown in Table 2 demon-

TABLE 2. *Enzyme-inhibiting activity of antiserum to purified A_2 (1957) neuraminidase against several strains of influenza A_2 virus.*

Virus	EI* titer of R301 antiserum
X-7(F1) (A_0E/57)	3000
Jap./305/57 (A_2E/57)	3000
Taiwan/64	500
Itsukaichi/65	400
Montevideo/67	520
England/67	50
Texas/68	25
Hong Kong/68	25

* Enzyme-inhibiting titer—reciprocal of serum dilution which reduces enzymatic activity of test virus by 50%.

strate a progressive loss of enzyme-inhibiting activity, the titer against the Hong Kong/68 strain being 100-fold less than against the homologous 1957 strain.

(b) *Cross-reactivity in hemagglutination-inhibition and enzyme-inhibition assays:* The antigenic relationships of hemagglutinin proteins among the seven viruses were measured by cross-reactivity of viruses and antisera to them in hemagglutination-inhibition tests. Although each antiserum reacted with every virus, appreciable differences in cross-reactivity of some pairs is evident (Table 3). In general, viruses most closely related temporally were also most similar with respect to hemagglutinin antigens. A similar comparison of the enzyme antigens of each of the viruses was made by titration of enzyme-inhibiting activity. In preliminary studies, we found that antihemagglutinin antibody could influence the results of these tests probably as a result of steric hindrance. Therefore, hybrid viruses containing the heterologous (non-cross-reacting) hemagglutinin of A_0/NWS and the enzyme of the test virus were utilized as the source of enzyme. Appreciable differences in cross-reactivity of the enzyme antigens were demonstrated (Table 4), but viruses antigenically different with regard to hemagglutinin were not necessarily dissimilar in their enzyme antigens. These relationships are shown more systematically in Table 5, in which similarity coefficients[16] of the seven viruses are indicated for both antigens.

Several generalizations can be made on the basis of the evidence presented in Table 5. Between 1957 and 1965, there was a relatively much greater antigenic change in neuraminidase than in hemagglutinin protein. During the interval

TABLE 3. *Antigenic relationships among seven influenza A_2 viruses as measured by cross-reactivity in hemagglutination-inhibition tests.*

	Viruses						
Sera	J.305 (1957)	Taiwan (1964)	Itsuk. (1965)	Mont. (1967)	Eng. (1967)	Texas (1968)	H.K. (1968)
Jap.305/57	*8192**	2048	2048	2048	1024	1024	64
Taiwan/64	1024	*2048*	4096	2048	256	512	64
Itsukaichi/65	2048	8192	*8192*	64	256	256	32
Montevideo/ 67	256	1024	2048	*1024*	512	1024	32
England/67	256	512	512	512	*8192*	2048	128
Texas/68	128	128	128	64	512	*1024*	64
Hong Kong/68	32	128	128	32	128	128	*4096*

* Homologous titers are in italics.

TABLE 4. *Antigenic relationships among seven influenza A_2 viruses as measured by cross-reactivity in enzyme inhibition tests.*

	Viruses Used as Enzyme Source						
Sera	A_0E (J. 305)	A_0E (Taiwan)	A_0E (Itsuk.)	A_0E (Mont.)	A_0E (Eng.)	A_0E (Texas)	A_0E (H.K.)
Jap.305/57	*2500**	125	425	375	35	25	35
Taiwan/64	525	*500*	425	275	225	100	125
Itsukaichi/65	2250	2200	*1875*	425	225	125	200
Montevideo/67	375	900	575	*3000*	2600	725	900
England/67	25	23	85	625	*525*	375	325
Texas/68	25	85	95	375	325	*275*	175
Hong Kong/68	20	25	17	425	125	175	*125*

* Reciprocal of serum dilution reducing enzymatic activity by 50%.

TABLE 5. *Similarity coefficients* of hemagglutinin and neuraminidase antigens of seven influenza A_2 viruses.*

	J.305	Taiwan	Itsuk.	Mont.	Eng.	Texas	H.K.
J.305/57	*1/1*	...	...	...	...	...	...
Taiwan/64	2.8/4.6	*1/1*	...	...	...	...	...
Itsuk./65	4/2.2	2/1	*1/1*	...	...	...	...
Mont./67	4/7.3	1/2.5	8/5.3	*1/1*	...	...	...
Eng./67	16/38	11.3/7.1	22.6/7.2	5.7/1	*1/1*	...	...
Texas/68	8/33	5.7/3.9	16/6.6	4/1.6	2.8/1.1	*1/1*	..
H.K./68	127/21.1	32/5.5	84.8/8.4	128/1	45.3/1.3	26.2/1.0	*1/1*

* Numerator = similarity coefficient of hemagglutinin antigens; denominator = similarity coefficient of enzyme antigens. Calculated from:

$$r = \sqrt{(\text{heterol. titer}^1/\text{homol. titer}^1) \times (\text{heterol. titer}^2/\text{homol. titer}^2)}$$ (ref. 16).

from 1965 to 1967–68, there appear to have been appreciable antigenic changes in both hemagglutinin and neuraminidase, the differences in hemagglutinin being more pronounced. Finally, the Hong Kong strain introduced during the summer of 1968 is markedly different from any earlier strains with respect to its hemagglutinin, but it possesses a neuraminidase antigen indistinguishable from the enzymes of strains isolated during the previous year.

(c) *Plaque-size reduction:* Antigenic differences in the neuraminidase proteins also were demonstrated by assay of plaque-size-reducing titers of antisera to the parent A_2 strains tested against hybrid viruses containing the enzyme but not the hemagglutinin of those strains. As shown in Table 6, antiserum to Jap. 305/57 virus has 16-fold more plaque-size-reducing activity against a virus with a homotypic enzyme than against a virus possessing the enzyme of the Hong Kong strain, while antiserum to the Hong Kong virus is 64 times more active against a hybrid virus with the Hong Kong enzyme than it is against a hybrid virus with the enzyme of Jap. 305 virus. The neuraminidases of Montevideo/67 and England/67 viruses are completely cross-reactive with each other and the enzymes of both are antigenically very similar to the enzyme of the Hong Kong virus as judged by plaque size reduction as well as enzyme-inhibition tests.

(d) *Further evidence of the novelty of the hemagglutinin of Hong Kong/68 virus:* Although the hemagglutinin antigens of the Jap.305/57 and Hong Kong/68 strains were very different by hemagglutination-inhibition tests, the two viruses and antisera to them did cross-react in low titer. Nevertheless, these two viruses possess much more closely related enzyme antigens than hemagglutinin antigens,

TABLE 6. *Plaque-size-reducing antibody titers of antisera to four viruses against recombinant viruses possessing the neuraminidase but not the hemagglutinin antigens of the same strains.*

	Viruses			
Antisera	X-7*	NWS E (Mont.)	NWS E (Eng.)	NWS E (H.K.)
Jap.305/57	*25,600*†	3,200	1,600	1,600
Montevideo/67	6,400	*51,200*	51,200	25,600
England/67	600	12,800	*12,800*	25,600
Hong Kong/68	800	25,600	25,600	*51,200*

* Recombinant virus prepared from A_0/NWS and A_2/RI/5$^+$/1957 (NWS E/57).
† Numbers in italics are homotypic titers.

and it appeared possible that some of the cross-reactivity apparent in hemagglutination-inhibition tests might actually be mediated by the antienzyme antibody rather than by the antihemagglutinin antibody. This possibility was investigated by measuring the cross-reactivity of Hong Kong/68 virus with other recombinants of the 1957 strain including: X-9 (A_2e) (see Table 1), a recombinant containing the 1957 A_2 hemagglutinin and the enzyme of A_0/NWS virus and X-15 (Equi E), which possesses the hemagglutinin antigen of Equine 1 virus and the enzyme of influenza A_2/1957 virus. The antigenic relationships of these viruses to Hong Kong/68 virus were tested in hemagglutination-inhibition and plaque assay determinations. The results (Table 7) demonstrate that all cross-reactivity between the 1957 strain and Hong Kong/68 virus is attributable to similarities of the enzymes. Antibody to X-9 virus which possesses the hemagglutinin but not the enzyme of the 1957 strain has no activity against the Hong Kong virus, and the Hong Kong antiserum is inactive in hemagglutination-inhibition and plaque-inhibition tests against X-9 virus. In addition, in hemagglutination-inhibition tests, there is more cross-reactivity between the Hong Kong strain and X-15, a hybrid virus possessing only the enzyme of A_2/1957 virus, than there is between the Hong Kong strain and the wild-type Jap.305/57 virus. When rabbit antisera to the other strains of influenza A_2 virus (Taiwan/64, Itsukaichi/65, Montevideo/67, England/67 and Texas/68) were tested by assay of plaque-inhibition against recombinant X-9 virus and Hong Kong virus, no plaque inhibition (antihemagglutinin antibody) against the Hong Kong virus was demonstrable at the lowest serum dilution which could be tested without nonspecific inhibition (1:800), although appreciable plaque size reduction (antineuraminidase activity) was evident. In contrast, these sera had plaque-inhibiting titers ranging from 1:3,200 to 1:51,200 against X-9 virus. These results provide additional evidence that the hemagglutinin of Hong Kong/68 virus is not only antigenically very different from Jap.305/57 virus but also has very little cross-reactivity with the hemagglutinin antigens of more recent A_2 virus isolates. They support the hypothesis that cross-reactivity of

TABLE 7. *Antigenic differences of the hemagglutinin antigens of A_2/1957 and Hong Kong/68 viruses: Influence of antineuraminidase antibody on hemagglutination-inhibition and plaque-inhibition determinations.*

	Antisera			
	Hong/Kong/68 (HK/68)	Jap.305 (A_2/1957/E-(1957))	X-9* (A_2/1957/e)	X-15† (Equi/1/E-(1957))
Hemagglutination-inhibition vs.:				
Hong Kong	*4096*‡	64	<16	128
Jap.305	32	*8192*	1024	128
X-9	<16	1024	*2048*	<16
X-15	128	64	<16	*4096*
Plaque-inhibition vs.:§				
Hong Kong	*102,400*	<800	<800	NT
X-9	<800	51,200	*51,200*	NT

NT, not tested.
* Recombinant virus containing A_2/1957 hemagglutinin, A_0/NWS (e) enzyme.
† Recombinant virus containing $Equi_1$ hemagglutinin, A_2/1957(E) enzyme.
‡ Homologous titers are in italics.
§ Reciprocal of serum dilution which reduces plaque number (plaque-inhibition) by 50%.

these viruses and Hong Kong/68 virus in hemagglutination-inhibition tests results from antigenic similarities of their neuraminidases.

(e) *Immunization studies in mice:* The influence of these antigenic relationships, demonstrated in *in vitro* and in tissue culture systems, has been investigated in parenterally immunized mice. In one experiment, mice immunized with either A_2/Jap.305/1957 virus or a hybrid virus containing the neuraminidase of the A_2/1957 strain but not its hemagglutinin (A_0E/1957), or with purified A_2/1957 neuraminidase had lower pulmonary virus titers and less extensive lung lesions after challenge with the mouse-adapted Hong Kong virus. The magnitude of the protection in mice immunized with the A_0E recombinant was equivalent to that observed in mice immunized with the wild-type A_2E virus. Furthermore, no protection resulted when mice were immunized with a hybrid virus containing the hemagglutinin of the A_2/1957 strain but not its enzyme (A_2e), although these animals were protected against challenge with A_2/Jap.305/1957 virus. In the same experiment, we also observed that immunization with neuraminidase derived from the A_2/1957 strain afforded considerably more protection against challenge with A_2/Jap.305/1957 virus than against Hong Kong/68 virus. These experiments support and extend observations made in *in vitro* and in tissue culture systems: There is no cross-reactivity between the hemagglutinin antigens of A_2/1957 virus and Hong Kong/68 virus, and antigenic differences of the enzyme proteins of these two strains are evident, but considerable antigenic cross-reactivity is still demonstrable. The only component of the 1957 virus which provided immunity against the Hong Kong strain was its neuraminidase.

(*Note added in proof:* These results were also confirmed by later experiments in which we found that only the neuraminidase component of a more recent A_2 virus isolate protected immunized mice against Hong Kong virus challenge.)

Discussion.—It is evident from these experiments that antigenic changes take place in both envelope proteins of influenza virus in nature and that changes may occur in one antigen without appreciable shift of the other antigen. The epidemiologic significance of major antigenic changes in viral hemagglutinin has been demonstrated repeatedly to be a major factor in cyclical outbreaks of pandemic disease. Whether or not antigenic changes in neuraminidase also provide a biological advantage for the virus is less certain, but it is probable that such mutations do not evolve to predominance at random.

In the period of 11 years since the first appearance of the A_2 strains of influenza virus, there appears to have been a sequence of antigenic changes in the two envelope proteins. The earliest changes were primarily in viral enzyme; there was then a period (1965 to 1967) during which there were antigenic changes of both proteins with more pronounced antigenic "drift" of hemagglutinin. Finally, with the appearance of the Hong Kong virus, there is evidence of mutation of great magnitude in the hemagglutinin antigen without concomitant antigenic change of the viral neuraminidase.

We propose that it is primarily the antibody directed to the enzyme component of earlier strains that provides protection against infection with the

Hong Kong virus. According to this view, the absence thus far of pandemic influenza on a scale comparable to events in 1957–58 is also a reflection of the modifying effects of the antineuraminidase antibody reactive with the enzyme of Hong Kong virus. Similar events may have occurred in 1947 with the introduction of the A_1 subtype which was antigenically dissimilar to the earlier A_0 strains with respect to its hemagglutinin, but cross-reactive with respect to its neuraminidase antigen.[9, 10] Although there were widespread epidemics in association with the appearance of the A_1 subtype, this antigenic change was not associated with pandemic influenza.

Although the arbitrary, antigenically determined subtype designations of human influenza A strains as A_0, A_1, and A_2 (representing chronologic periods of prevalence) have been operationally useful, the foregoing observations suggest that they may be misleadingly simplistic. In our laboratory the hemagglutinin antigens of the NWS and Bel strains of the A_0 subtype differ more one from the other than do those of the NWS (A_0) and the CAM (A_1) strains. It is clear that a rational taxonomy of the influenza A viruses will be dependent upon antigenic analyses of both surface antigens. Furthermore, to preclude antibody to one antigen from influencing titrations of antibody to the other, it may be necessary to employ hybrid viruses in which neuraminidase and hemagglutinin antigens have been segregated.

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